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FORMATION OF THE CARBOHYDRATE MOIETIES IN THE GLUCOAMYLASE OF *ASPERGILLUS NIGER*

by

Irwin Jon Russell

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TITLE

Formation of the Carbohydrate Moieties in the Glucoamylase of Aspergillus niger

BY

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INTRODUCTION

Glucoamylases (α-D-(1→4)-glucan glucohydrolases, E.C. 3.2.1.3) are a group of enzymes capable of hydrolyzing starch to glucose. They are produced by several species of fungi, bacteria and yeasts. A relatively broad specificity is indicated by their ability to hydrolyze α-D-(1→4)-, α-D-(1→6)- and α-D-(1→3)-linkages in glucans. Maltose, nigerose and isomaltose are all substrates for the enzyme having relative rates of hydrolysis of 30:2:1, respectively. The only product in each case is D-glucose. A similar enzyme has been detected in animal tissues and is referred to as γ-amylase. Its mode of action is the same as the fungal glucoamylases and it is capable of converting glycogen directly to glucose. The fungal enzymes are used commercially for the production of glucose syrups from starch and are also currently being investigated in humans as a treatment for glycogen storage disease.

The glucoamylase produced by Aspergillus niger is an extracellular enzyme system composed of two isoenzyme forms. These isoenzymes are glycoproteins and are similar in their hydrolytic activity, but differ in some physical properties including molecular size and electrophoretic mobility. The isoenzyme migrating more rapidly toward the anode during paper electrophoresis is designated glucoamylase I and the second, glucoamylase II. The

carbohydrate moieties are composed of D-mannose, D-galactose and D-glucose.

The production of the A. niger glucoamylase is induced by maltose, starch and a number of glucans. The rate of synthesis of these enzymes is dependent upon the nature of the carbon and nitrogen sources, as well as the concentration of these substrates within the growth media. The enzymes are released at a time when the organism has passed the most rapid stage of cell growth. Glucoamylase does not appear to be stored intracellularly or to exist in a proenzyme form.

The function of the carbohydrate moieties in the fungal glucoamylases is unknown. In addition, there is currently no information available concerning the pathways involved in incorporation of the carbohydrate into the enzyme. The biosynthesis of proteins and polysaccharides has been studied to a considerable extent in recent years and, as a result, convincing evidence supports current concepts for these processes. More recently, study has been directed at the mechanisms involved in the formation of glycoproteins.

It was the purpose of this study to begin an investigation of the pathways and mechanisms leading to the incorporation of the carbohydrate residues into the glucoamylase from A. niger. A multi-step procedure, including ion-exchange chromatography and gel filtration, was used to obtain purified glucoamylase I and II from culture filtrates of A. niger grown on mannose-1-^{14}C or glucose-

1-^{14}C. Homogeneity of the purified enzymes was determined by disc and paper electrophoresis. The free neutral hexoses were isolated from the purified glycoprotein by acid hydrolysis and separated by paper chromatography. These hexoses were oxidized with hypoiodate and degraded with periodate. The distribution of radioactivity in carbon atoms one and six of the hexoses was determined..

Nucleotide sugars produced by A. niger were studied by isolation from intact mycelia and by in vitro synthesis.

LITERATURE REVIEW

The glycoprotein nature of a group of fungal carbohydrases has been established by numerous investigations over the past decade. These enzymes are extracellular secretions and conform to the definition of a glycoprotein proposed by Gottschalk (1). They are primarily protein in nature but contain carbohydrate covalently attached to the polypeptide chain. The carbohydrate moieties in these carbohydrases are composed primarily of neutral sugars which include D-mannose, D-glucose and D-galactose (2). Less commonly, D-xylose and D-glucosamine are also present (3). Carbohydrate contents ranging from 2-16% have been reported (2-6) for enzymes of this group.

The glucose oxidase from Aspergillus niger was reported (2,4) to contain D-mannose (14%), D-galactose (0.3%) and D-glucosamine (2.3%). Swoboda and Massey (5) reported the percentage of carbohydrate in glucose oxidase from the same source to be 16.5 (±0.8%), in good agreement with the previous data. From a third preparation of glucose oxidase from A. niger, Nakamura and Fujiki (6) found the same amount of carbohydrate but, in contrast to the data above, glucose was present and not galactose. The values were D-mannose (13.2%), D-glucose (0.7%) and hexosamine (2.1%). These same workers determined the comparable values for the glucose oxidase from Penicillium amagasakiense

to be D-mannose (8.3%), D-glucose (1.1%) and hexosamine (1.7%). Mild periodate oxidation of purified glucose oxidase resulted in only 15% loss of enzymatic activity when 50-60% of the carbohydrate had been oxidized (7). This suggests little or no involvement of the carbohydrate moieties with the active site of the enzyme or with maintenence of the active tertiary structure.

The α-amylase from A. oryzae also has been demonstrated to be glycoprotein in nature (8). Hanafus et al. (3) showed that the carbohydrate portion of the enzyme was not dissociated by ion-exchange chromatography, paper electrophoresis or by three recrystallizations. Using a commercial preparation of Taka-amylase A, these workers found 2.73% carbohydrate consisting of mannose, xylose and hexosamine. Based on a molecular weight of 53,000, a composition of 8 residues of mannose, 1 residue of xylose and 2 residues of hexosamine per mole of enzyme was reported. In contrast, an enzyme which was produced by growth of the organism in the laboratory had the same specific activity as the commercial preparation but contained only 0.25% carbohydrate. One of the conclusions from this data was that the carbohydrate was not necessary for enzymatic activity. A more recent investigation (9) has established that the hexosamine is 2-acetamido-2-deoxy-D-glucose and that the carbohydrate is linked to the polypeptide via an N-glycosidic bond between this hexosamine and the amide group of asparagine; a 2-acetamido-1-β-(L-aspartamido)-

1,2-dideoxy-D-glucose structure being most likely.

Glucoamylases (α-D-(1→4)-D-glucan glucohydrolase, E.C. 3.2.1.3) are reported to be produced by fungal sources such as Endomycopsis capsularis (10), A. niger (11), A. oryzae (12), A. phoenicis (13) and Rhizopus delemar (14); by certain yeasts and bacteria including Saccharomyces diastaticus (15) and Clostridium acetobutylicum (16). An enzyme having similar activity has been detected in animal tissues, particularly in the liver and in lesser amounts in other organs (17,18). The glucoamylase from A. niger is probably the most completely studied of the above sources. It has been reported to consist of two isoenzymes (2,11) which have been designated glucoamylase I and II according to their electrophoretic mobility (19). Pazur (2) reported the carbohydrate composition of the A. niger isoenzymes as indicated in Table I. Using a different preparation and analytical method, Horner (20) found a lower

TABLE I

Carbohydrate content of glucoamylases from A. niger

	Percentage composition by weight		
	D-Mannose	D-Glucose	D-Galactose
Pazur			
Glucoamylase I	8	2	0.2
Glucoamylase II	15	3	0.2
Horner			
Glucoamylase I	6.8	2.1	0.3
Glucoamylase II	10.6	4.4	0.7

total carbohydrate content and a slightly different distribution of the individual sugars. It should be pointed out that the differences may be related to the quantitative method of analysis used and to destruction of the hexoses during the analytical procedure, in addition to possible variations in the actual protein composition.

In contrast, the glucoamylase from R. delemar contains about 13% carbohydrate, primarily D-mannose and D-glucosamine. The hexosamine accounts for about 25% of the total carbohydrate and in all likelihood exists within the native enzyme in the N-acetyl form (14). To date, there has been no report of an amino sugar in the A. niger enzymes. The glucoamylase from A. phoenicis has been purified and found to consist of two forms which are glycoprotein in nature (13). As with the glucoamylase from A. niger, both forms of the glucoamylase from A. phoenicis were found to contain D-mannose as the predominant hexose with lesser amounts of D-glucose and D-galactose. No hexosamine or sialic acid were detected.

Eylar (21) has proposed an explanation for a finding that most extracellular proteins contain bound carbohydrate, which is virtually absent from intracellular proteins. Since there appears to be no specific role for the carbohydrate present, he suggests that it acts as a chemical label which, upon interaction with a membrane receptor or carrier, promotes the transport of the newly synthesized glycoprotein into the extracellular environment. "It is

predicted by this hypothesis that the carbohydrate need play no functional role in biologically active proteins, that the carbohydrate unit in glycoproteins from the same cell would be identical or very similar, and that glycoprotein biosynthesis would involve similar primary or tertiary structures at those regions in the protein molecules where the carbohydrate is attached." (21) In support of this hypothesis, Eylar lists 97 extracellular proteins from mammalian sources which are glycoproteins and tabulates their carbohydrate composition. An additional 15 extracellular proteins listed have not been shown to contain carbohydrate. Finally, except for one case, the 20 intracellular proteins studied appeared to lack any associated carbohydrate. An alternative role for the carbohydrate moieties in glycoproteins could be to increase the stability to denaturation. This property appears to be related to the amount of carbohydrate present (22). Glycoproteins, such as ovalbumin and fibrinogen, in which the carbohydrate comprises only about 3% of the total, are easily denatured. However, mucopolysaccharides, such as the blood group substances, in which the carbohydrate content is very high, do not seem to undergo denaturation.

In view of the variation of the carbohydrate composition in the fungal glucoamylases, their commercial importance, and the lack of information concerning the function of the carbohydrate moieties, it became of increasing interest to study the biosynthesis of proteins containing bound

sugar. Since the amino acid sequence is probably determined by the same mechanisms which are responsible for the primary structure of proteins in general (1,23), the study of glycoprotein synthesis can be directed to the pathways involved in the attachment of the carbohydrate. There is at present no information available on this process for the fungal carbohydrases. Thus it will be the purpose of this review to first discuss the data from other glycoprotein synthesizing systems which establish a basic model for the incorporation of carbohydrates into glycoproteins and then to present information which has a direct bearing on the production of the glucoamylase enzymes by A. niger.

Roseman (24) has proposed two possible mechanisms by which carbohydrate may be incorporated into a covalently linked protein-polysaccharide complex. In the first mechanism, the oligosaccharides are linked to specific amino acids prior to activation of the amino acid and subsequent incorporation into the growing polypeptide chain. The second mechanism suggest that the carbohydrate is attached to the polypeptide only after the polypeptide chain is completely synthesized and has moved to a different site within the cell.

To distinguish between these alternatives, Sarcione et al. (25) administered radioactively labeled galactose and glucosamine to actively metabolizing rat liver skin and examined the incorporation of the label into glycoproteins within various liver cell fractions with time. Leucine-^{14}C

was also administered as a measure of the rate of general protein synthesis. If the oligossacharides were incorporated into the growing peptide chain at any step prior to completion of polypeptide synthesis, the specific activity of ribosomal protein-bound carbohydrate would be expected to be higher and would reach its maximum earlier than that in other subcellular protein fractions. If, on the other hand, carbohydrate is attached at specific sites along the completed peptide chain after release from the ribosomes, then the specific activity of protein bound carbohydrate would be expected to be minimal in the ribosomal protein. In the latter case, it would increase most rapidly at the actual site of carbohydrate attachment.

The subcellular fractions that these workers studied (25,26) were soluble protein, ribosomal protein, and deoxycholate-soluble protein. The effect of deoxycholate according to Palade and Kiekevitz (27) is "to disrupt the vesicles of the endoplasmic reticulum found in the microsome fraction of liver and to solubilize their limit membranes while leaving the ribosomes apparently intact." The incorporation of leucine-^{14}C showed a rapid increase in specific activity within the ribosomal protein up to 3 minutes and then a decrease in specific activity to a relatively low level by 7 minutes. The amount of label being incorporated into the deoxycholate soluble fraction showed a regular increase but lagged behind that for the ribosomes. This fraction did not reach its maximum within the 7-minute

study period. Little or no activity was observed in the soluble protein. When galactose-1-^{14}C was used the results were quite different. The deoxycholate soluble protein showed a rapid increase in specific activity until three minutes, after which the specific activity continued to rise but not at as high a rate. The specific activity of the other protein fractions studied was negligible.

Similarly, in a double-labeling experiment (25) the patterns of leucine-^{3}H and glucosamine-^{14}C incorporation into protein were strikingly different. Initial incorporation of leucine-^{3}H was into the ribosomal protein; this was followed by its progressive appearance in the deoxycholate-soluble protein. In contrast, incorporation of glucosamine-^{14}C was most rapid in the deoxycholate-soluble fraction, and virtually no incorporation was observed into the ribosomal protein. To provide further evidence that the glucosamine-^{14}C isolated from a 15-minute deoxycholate-soluble protein fraction of microsomes was in fact incorporated into the protein, glucosamine was isolated from glycopeptides obtained from pronase digests of the deoxycholate-soluble fraction. The radioactivity was shown to be inseparable from a number of glycopeptides each containing four amino acids. In addition, when the radioactivity was released by hydrolysis it had a specific activity comparable to glucosamine obtained by hydrolysis of the unfractionated deoxycholate-soluble protein.

It would thus seem clear that this evidence supports

the second mechanism proposed by Roseman (24). The protein is synthesized on the ribosome and the completed polypeptide is released. The polypeptide is then retained in some manner by the membranous matrix of the endoplasmic reticulum (deoxycholate-soluble fraction) where the carbohydrate is covalently attached.

Data obtained by Spiro and Spiro (28) indicate that the monosaccharides may be added sequentially rather than as an oligosaccharide unit. Microsomal particles from calf thyroid were incubated with amino acids and sugars in vitro with appropriate media and the deoxycholate-soluble protein collected. The major component was shown to be identical with authentic thyroglobulin by paper electrophoresis and immunochemical analysis. Various ^{14}C-labeled sugars and puromycin were added simultaneously to identical incubation mixtures. Under these conditions, protein synthesis was completely inhibited while the incorporation of the various carbohydrates into the deoxycholate-soluble thyroglobulin was observed to depend upon the sugar concerned. Relatively, the extent of the inhibition of ^{14}C-incorporation into deoxycholate-soluble protein was mannose>glucosamine>galactose>sialic acid. It should be noted that there is a relationship between the degree of inhibition of incorporation of the various sugars and their sequential arrangement in the carbohydrate polymers (29) with the most peripherally located sugar, sialic acid, being least affected.

Yu-Teh et al. (30) investigated glycopeptides obtained

from the deoxycholate-soluble fraction from rat liver microsomes. The carbohydrate sequence was determined to contain sugar residues which occupy the inner core of the completed plasma glycoprotein (21,31). The galactose and sialic acid residues which are located at the non-reducing end of the carbohydrate fragment were absent. It was suggested that these two missing carbohydrate moieties would be sequentially added at some time before the release of the glycoprotein from the cell. These data would be consistent with a stepwise attachment of the sugar components to the polypeptide (28).

Robinson, Molnar and Winzler (32,33) reported the incorporation of glucosamine-^{14}C into blood glycoprotein and into both 10% trichloroacetic acid (TCA)-soluble and insoluble fractions from rat liver after the radioactive sugar was injected into the live animal. The specific activity of a 10% TCA-soluble liver extract increased very rapidly to a maximum and then declined. Its decline was concomitant with an increase in the specific activity of the 10% TCA-insoluble fraction, which in turn reached a maximum and then decreased as the specific activity of the blood glycoprotein increased. These workers concluded that the glycoproteins represented by the 10% TCA-insoluble fraction were released into the blood as rapidly as they were produced in the liver cell microsomes. That is, there appeared to be no build-up or storage of them within the cell. Attempts were then made to determine the identity of the radioactive components in the 10% TCA-soluble fraction (34). It was possible

to obtain larger quantities of label within the soluble fraction by inhibition of protein synthesis with puromycin. Significantly, under these conditions, UDP-N-acetylglucosamine was found, together with some of its metabolic products which have been previously reported (35,36).

Since that time, the transfer of sugars from sugar nucleotides to polypeptide chains has been well documented (37-42). Hagopian and Eylar (37) studied the action of the UDP-N-acetylgalactosaminyltransferase from bovine submaxillary glands and observed that it catalyzed the transfer of ^{14}C-labeled N-acetylgalactosamine to a basic myelin protein, normally free of carbohydrate groups, from bovine spinal cord. The natural receptor, a bovine submaxillary glycoprotein, served again in that capacity following digestion with sialidase and hexosaminidase. The receptor activity of the myelin protein was 25% that of the carbohydrase-treated glycoprotein. Receptor activity was substantially reduced by pronase digestion of the carbohydrate-free myelin protein. However, 50% of the receptor activity was retained even after 5 minutes at 100° or treatment with 8 M urea. The carbohydrate was shown to be O-glycosidically linked to the hydroxyl group of threonine. The enzyme used in this case was a partially purified system which required manganous ion for activity.

McGuire *et al.* (38), working with a similar system partially purified from goat colostrum, also observed the transfer of N-acetylgalactosamine from a nucleotide sugar

to orosomucoid which had been previously treated with sialidase and β-galactosidase. UDP-N-Acetylgalactosamine, UDP-N-acetylglucosamine and UDP-glucose were all substrates for the transferase. Sarcione and Carmody (39) also showed that the galactosyltransferase in rat liver could be solubilized from the particulate microsomal fraction without loss of activity by treatment with deoxycholate which greatly was capable of transferring the sugar moiety from UDP-galactose-^{14}C to protein receptors including polypeptides still bound to ribosomes. The latter had been separated from the microsomal fraction by the deoxycholate solubilization step. This receptor activity was not destroyed by heating the ribosomes at 90^{o} for 5 minutes. Neither galactose-^{14}C nor galactose-^{14}C 1-phosphate was incorporated into protein under these conditions. After proteolytic digestion of the glycoprotein synthesized in the presence of UDP-galactose-1-^{14}C, galactose was shown to contain all the radioactivity. The amino acids present in two small glycopeptides isolated from this digestion were aspartic acid, glutamic acid, serine, alanine and ornithine. Transferase enzymes have also been reported for CDP-sialic acid (40), UDP-xylose (41), UDP-galactose (42) and UDP-glucosamine.

Peterson and Leblond (43) injected glucose-^{14}C and $Na_2{}^{35}SO_4$ subcutaneously into rats and prepared radioautograms of thin slices of tissue which had been actively synthesizing glycoprotein. These studies indicated that protein was

made in the rough region of the endoplasmic reticulum and that the majority of the carbohydrate was attached only after the polypeptide chain had migrated into the Golgi region.

Thus the available evidence supports the following hypothesis for the biosynthesis of a glycoprotein in a mammalian system. The polypeptide chain is produced in the manner of other proteins. As the completed polypeptide is released from the ribosomes and migrates into the smooth endoplasmic reticulum, carbohydrate is added, a unit at a time, by its transfer from a nucleotide sugar. The addition of carbohydrate is completed near or within the Golgi bodies followed by release of Golgi body vesicles which migrate to the surface and deposit their contents outside the cell (43,44).

While it might appear by analogy that the biosynthesis of fungal glycoproteins would follow the same pattern as has been demonstrated for mammalian systems, this has not yet been verified. However, there is information available concerning the factors which control the production of these proteins, particularly for the glucoamylases. Employing an adenine-requiring strain of A. niger and a nitrogen source of ammonium nitrate and glutamate, Okazaki and Terui (45) reported that the highest levels of glucoamylase resulted with maltose as the carbon source. Lesser amounts were observed when starch, dextrin or glycogen were used. A number of other carbon sources such as glucose, galactose and

sorbose resulted in lower enzyme levels than with maltose, while fructose, mannose, and mannitol did not yield that enzyme even though mycelial mass was formed.

Using a different assay procedure for glucoamylase, Lineback, Georgi and Doty (46) observed that A. niger NRRL 330 grown on mannose produced glucoamylase. Enzyme production varied with the nature and concentration of both the nitrogen and carbon sources. In a more recent study, Barton, Lineback and Georgi (47) reported that glucoamylase activity in the culture media showed marked increases after 3 days and after 6 days of growth. Of the nitrogen sources studied, corn steeping liquor and nutrient broth (2.5%) resulted in the highest levels of enzyme activity. Of the carbon sources studied, glucose was most efficient for production of glucoamylase while growth on mannose resulted in about half that quantity of enzyme. Carbohydrate levels of 1.0 or 2.0% were found to produce higher enzyme yields than 5.0%. It has been suggested (48,49) that the synthesis of glucoamylase I and II is controlled by different genes and that they are produced sequentially by the organism. However, in the above study, results indicated that both enzymes were present after three days as well as after six days with a glucose-yeast extract media. Nevertheless, the double gene concept is supported by the fact that only glucoamylase I was produced by the organism in a glucose-ammonium chloride media (47). Evidence obtained by Kawamura and Sawai (49) indicates that the glucoamylases of Candida pelliculosa